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(57) Abstract

Human monoclonal antibodies of predetermined specificity are produced by isolating human B lymphocytes of a given specificity and transforming them with Epstein-Barr Virus. Similarly, human monoclonal antibodies of a predetermined class can be produced by transformation of B lymphocytes selected for given surface Ig heavy chains (γ, α and μ). After culturing tranformed B lymphocytes in limiting dilution and cloning the selected cell(s), the resulting clones secrete human monoclonal antibodies which specifically bind to selected antigen(s).

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PREPARATION OF HUMAN MONOCLONAL ANTIBODIES OF SELECTED SPECIFICITY AND ISOTYPES

BACKGROUND OF THE INVENTION

The first process for producing monoclonal antibodies was demonstrated by Kohler and Milstein in 1975 (Nature, 256: 495-497 (1975)) and involved the fusion of mouse myeloma cells to spleen cells from immunized mice. The resulting hybridomas produced large quantities of antigen-specific monoclonal antibodies. The success of this method is largely due to the lack of any restriction to repeated immunization of animals and the ease in obtaining non-human spleen cells.

Many variations of the Kohler-Milstein immunology-revolutionizing technique have been disclosed, each attempting to improve the technique or to produce human monoclonal antibodies (see Patent Nos. 4,529,694; 4,550,086; 4,443,427; and 4,361,549). One method which attempts to apply the Kohler-Milstein method to the production of human monoclonal antibodies is disclosed in Patent No. 4,608,337, relating to the production of human antibodies by a hybridoma cell formed by fusing a human myeloma cell with a human B cell.

Researchers have been faced with several problems in producing human monoclonal antibodies using the Kohler-Milstein technique or a process similar to those described in the above-noted patents. First, in an animal system, enrichment of antibody-producing cells is accomplished by hyperimmunization of an animal (usually, a rabbit or mouse). Actively immunizing humans with certain antigens is often impossible, especially with certain foreign or toxic antigens.

Human spleens or spleen cells are most difficult to obtain, and animal spleen cells are limited in their desirability for use in vivo in humans due to their animal or "foreign" heritage.

Finally, all of the processes noted above use a myeloma cell as a fusion partner for the immortalization of the B lymphocytes. In the mouse system, fusion of human B lymphocytes with mouse myeloma cells causes segregation of the human chromosomes. In the human system, fusion of human B lymphocytes with human myeloma cells exhibits poor growth in culture, and the lack of good human cells for fusion partners (myeloma cells), the low frequency of fusion events, and the relative paucity of circulating B lymphocytes with defined specificity (in humans, who can not be immunized with certain antigens) have made the production of human monoclonal antibodies most difficult.

The present invention overcomes these problems in a relatively simple, two step in vitro procedure for producing human monoclonal antibodies from human B lymphocytes (or B cell isotypes), without an in vivo active immunization of humans. It overcomes the problem of hyperimmunization by taking advantage of the fact that immunoglobulin molecules with specific antigen binding affinities are found on the surface of B lymphocytes present in the normal human repetoire. Based on isotype and/or antigen specificity, these cells can be be isolated and enriched by fluorescence-activated cell sorting (FACS). The present invention overcomes the problem of using spleen cells by employing peripheral blood B lymphocytes, easily obtainable from human blood. The present invention overcomes the problems associated with immortalization of B lymphocytes with myeloma cells by immortalizing human peripheral lymphocytes by transformation with a virus, preferably Epstein-Barr Virus (EBV). Furthermore, the plating efficiency and stability of the EBV-transformed cell lines can be increased by fusion with a human fusion partner.

One aspect of the present invention involves selecting B-lymphocytes which bind to a predetermined, purified antigen and infecting the desired lymphocytes with Epstein-Barr Virus. The transformed (infected) lymphocytes, once cultured under certain conditions, produce human monoclonal antibodies to the predetermined antigen.

Another aspect of the present invention involves selecting B lymphocytes bearing surface receptors to the γ, α, or μ heavy chain isotype using isotype-specific antibody probes. The isotype specific cells are then amplified by transformation with EBV, cultured under certain conditions, and cloned in order to produce cell lines which produce human monoclonal antibodies of a predetermined class (IgG, IgA, and IgM) and of the desired specificity.

Another aspect of the present invention involves the inherent advantage of using human B cells -- they can be obtained from subjects with ongoing pathological processes (wherein the subject produces antibodies to a disease). These particular B cells, therefore, are specific for cells of a specific disease.

Another aspect of the present invention is the production of human monoclonal antibodies useful in therapeutic procedures. Human monoclonal antibodies are produced for the treatment of viral infections including, but not limited to AIDS, rabies, and tetanus. Some of the human monoclonal antibodies of the present invention are useful in treating bacterial diseases, tumors, and immune modulations (wherein the human monoclonal antibodies are specific for subsets of human T-cells).

Another aspect of the present invention is the production of human monoclonal antibodies useful in diagnostic protocols. It is well known to use monoclonal antibodies (of animal origin) in imaging tumors, abscesses, and the like, and for use in diagnostic techniques such as positron emission topography. The human monoclonal antibodies of the present invention can be substituted for animal monoclonal antibodies, thus eliminating the need for administering a "foreign" reagent, and providing antibodies generated from a human's natural response.

Another aspect of the present invention is the production of human monoclonal antibodies to the Leu-1$^+$ subset of B lymphocytes, human autoantibodies also referred to as "natural" antibodies.

Another aspect of the present invention is the production of human monoclonal antibodies to certain tumors using specific tumor-generated B cells. Patients with various autoimmune diseases and tumors may now be treated with human monoclonal antibodies generated from the tumor or disease cells themselves. Examples of tumors include, but are not limited to colon carcinoma, lung cancer, and mammarian carcinoma. Examples of autoimmune diseases include, but are not limited to Rheumatoid arthritis, systemic lupus erythematosus, thyroiditis, and insulin-dependent diabetes mellitus.

Another aspect of the present invention is the production of human monoclonal antibodies generated from B lymphocytes obtained from humans previously actively immunized with a foreign antigen. Examples of such antigens include, but are not limited to tetanus, mumps, and whooping cough.

Another aspect of the present invention is the production of "polyreactive" human monoclonal antibodies. These antibodies are implicated in human autoimmunity, the scavenger system, and the natural defense system. Some of these antibodies have been described in Notkins, et al., Ann. N.Y. Acad. Sci., 475:123-134 (1986) and Casali, et al., "In Multiple Specific Antibodies", International Reviews of Immunology, eds. H. Kohler and C. Bona, New York, Harwood Academic Publishers (1987).

Another aspect of the present invention is the production of anti-idiotypic antibodies (anti-anti-antibodies) useful diagnostically for identifying the same antibody in different individuals (thus recognizing individuals with the same disease). See, for example, Uchigata, et al., J. Immunol., "Human Monoclonal Multiple-organ Reactive Autoantibodies Distinguished by Mouse Monoclonal Anti-Idiotypic Antibodies: Expression of Idiotopes in Humans With and Without Autoimmune Diseases", in press.

B-lymphocytes, representing 5-15% of the circulating lymphoid pool, are classicly defined by the presence of endogenously produced immunoglobulins -- they are an antibody-producing class of lymphocytes involved in the immune system of humans and animals. When a "foreign" substance enters the body, receptors (immunoglobulin molecules) specific for the foreign substance are expressed on the surface of B-lymphocytes as part of the normal immune response. B lymphocytes are therefore capable of making antibodies with a desired specificity, i.e., as a normal response to the antigen which has invaded the body.

The key to the present invention makes use, in part, of the B lymphocyte's ability to express specific receptors for antigens. The key to the process, therefore, is the isolation of a B-lymphocyte bearing certain immunoglobulin molecules on its surface. In the present invention, B-cells capable of making antibody with the desired specificity can be separated from irrelevant B cells by using the antigen in question as a probe. Immunofluorescence tests can be used to identify the probe after it binds to the B-lymphocytes. Accordingly, the antigen is fluorescently tagged, incubated with B-cells, and put through a fluorescent activated cell sorter (FACS), a machine which measures the fluorescence intensity of each cell. The cells are then separated according to their particular fluorescent brightness. Positively-selected cells (high fluorescence) are then transformed with EBV and propagated in culture.

Similarly, B lymphocytes bearing surface antigens of the γ, α, or μ heavy chain isotype can be isolated by FACS sorting using isotype-specific monoclonal antibodies.

Under these conditions, B lymphocytes from normal individuals can be used to generate clones secreting human monoclonal antibodies of selected antigen specificity.

DESCRIPTION OF THE FIGURES

Figure 1 shows FACS analysis and sorting of human B lymphocytes reacting with biotinylated human thyroglobulin (A), and tetanus toxoid (B). Panel (C) shows FACS analysis of EBV-transformed cells (from a clone of the invention) incubated with biotinylated thyroglobulin and FITC-avidin, or with FITC-avidin alone.

Figure 2 shows detection of thyroglobulin or tetanus toxoid binding antibodies in fluids from microcultures containing selected B lymphocytes infected with EBV.

Figure 3 shows the FACS analysis and sorting of human peripheral blood B lymphocytes based on heavy chain isotypes. The solid lines in panels A, D and G represent B cells reacted with FITC-goat $F(ab')_2$ fragment to human heavy chains IgG, IgA and IgM, respectively.

Figure 4 shows the antibodies produced by isotype-selected B lymphocytes. Each dot represents the concentration of antibody (expressed as absorbance at 492 nm) in the culture fluid from a single microculture well.

Figure 5 shows antibodies produced from human Leu-1^+ B lymphocytes.

SPECIFIC DESCRIPTION OF THE INVENTION

The method of preparing the human monoclonal antibodies generally comprises the following steps:

a) Isolating human B lymphocytes of defined specificity. One skilled in the art will recognize that there are several procedures designed to ioslate particular B lymphocytes. One method included within the scope of the present invention involves incubating labeled or unlabeled purified antigens with human peripheral blood B lymphocytes. Another method, disclosed below, involves isolating B lymphocyte isotypes using monoclonal antibodies which specifically bind to the desired isotype.

The antigens are purified according to well known techniques, and vary according to the antigen used (both foreign antigens and autoantigens can be used in the present invention). Two methods -- for tetanus toxoid and thyroglobulin -- are shown in the Examples.

The purified antigen may then be labeled. Labeling antigens is also well known by practitioners in the art. One such labeling procedure uses biotin or a biotin compound, a low molecular weight marker which provides reproducible labeling, but does not alter the antigen's binding capacity. One of the benefits of the present invention is that biotinylation of even small peptides does not usually interfere with the binding capacity of the antigen, due to the relatively small size of the biotin molecule (approximately 341 daltons). Those skilled in the art will recognize that other labeling procedures may be used in the practice of this invention; these procedures are included within the scope of the present invention.

Another method of isolating B lymphocytes of the desired specificity is by using unlabeled antigens and the identifying and isolating of the desired B lymphocytes by the limiting dilution method.

The B-lymphocytes are purified from peripheral blood of healthy human donors. Those skilled in the art will recognize that many methods exist for purifying B-lymphocytes. One method involves placing blood from healthy human donor on a gradient medium for separating lymphocytes. Monocytes are removed from other mononuclear cells by two cycles of incubation at 37°C in plastic 150-cm^2 tissue culture flasks, and/or by incubation with iron carbonyl particles and subsequent removal of iron-loaded monocytes by a magnet. This mononuclear fraction is then incubated in ice with AET (2-aminoethylisothioronium bromide hydrobromide)-treated sheep red blood cells (SRBC) to allow for rosette formation. Non-erythrocyte (E) rosette-forming cells were recovered after application of the whole SRBC mononuclear fraction to a lymphocyte-separating medium gradient. This non-adherent non-E rosetting fraction contained

at least 50% B cells, less than 1% monocytes, and variable amounts of lymphocytes with the natural killer (NK) phenotype. As defined in the present invention, this non-E rosetting B-enriched lymphocyte fraction is referred to as B lymphocytes or B-cells.

Those skilled in the art will recognize that many methods exist for incubating an antigen with a B-lymphocyte. One such method is disclosed in the Examples.

b) Selecting the lymphocytes to which the antigens bind by fluorescence-activated cell sorting (FACS). Positively-selected (high fluorescence) cells are immortalized by, in the preferred embodiment, transformation with Epstein-Barr Virus (EBV), and grown in microculture wells.

The selection process preferred in the present invention takes advantage of the extraordinarily high binding affinity of avidin for biotin. Human B cells incubated with biotinylated antigen as noted above, are washed and then reacted with fluorescein isothiocyanate (FITC)-avidin (1.56×10^{-9}M). After washing with bovine serum albumin, cells are resuspended at a density of 10^6 cells per milliliter in medium and applied to a Becton and Dickinson 440 FACS with an Argon 466 laser.

Approximately 7% of the cells incubated with biotinylated antigen and FITC-avidin display a higher degree of fluorescence than a control (B-cells incubated with FITC-avidin alone). The lymphocytes which bound the biotinylated antigen are sorted, with the positive fraction (high fluorescence) and a negative fraction (low fluorescence intensity) separated for transformation with EBV.

5×10^5 B cells from each fraction are infected with about 5×10^5 transforming units of EBV and then distributed in microcultures. EBV used to infect the B cells can be obtained from culture fluids of B95-8 marmoset lymphoma cells incubated at 37°C in the presence of 1.62×10^{-8} M 4-phorbol 12b-myristate 13a-acetate (TPA, Sigma). This virus preparation has a titer of 5×10^5 transforming units per milliliter, one unit being the minimum amount of virus capable of transforming 10^4 purified-

human B cells.

c) Culturing positively-selected EBV-transformed cells, and cloning them in limiting dilution. The culturing and cloning procedures are well known to practitioners in the art; the techniques described below are illustrative, and are not intended to limit the scope of the invention thereby.

The positively-selected EBV-transformed cells are resuspended and distributed at 4000 cells per well into a 96-well U-bottom culture plate containing 5 x 10^4 irradiated (2500 rads) syngeneic human peripheral blood mononuclear cells as a feeder layer. After about 18 days of incubation, culture fluids are harvested and assessed for antibody content. As shown in the Examples, all of the wells from the positively sorted fraction produced high concentrations of antibody. These cells were cloned in limiting dilutions at ten, five, two, and one cells per well in the presence of an allogeneic-irradiated feeder layer. Cell lines generated after three cloning steps produced amounts of antibody ranging from 62-12000 ngeq/ml. These antibody-producing clones were stable for a number of months. Repeated cloning of the cells helped to maintain antigen-specific antibody production. Fusing the cells with non-secreting human cell line also increases the stability of the clone. See Example 6.

Most of the clones produced antibodies of the IgM class, an expected result since IgM-bearing B cells constitute the majority (> 95%) of the circulating B lymphocytes in normal subjects. As noted in the Examples, however, clones producing IgG antibodies were generated from blood of patients with Hashimoto's thyroiditis (Table 1), and clones producing IgG antibodies to insulin and tetanus toxoid were generated from peripheral blood of patients with Type I (insulin deficient) diabetes and subjects recently immunized with TT, respectively. The IgM-producing clones (from non-immunized subjects) seem to represent silent, most likely virgin, B cells present in the normal circulating lymphoid pool; these silent cells can then be activated by in vitro in EBV. In contrast, the IgG-producing B cells from

patients with autoimmune thyroiditis or the IgG-producing B cells from patients recently vaccinated with tetanus toxoid probably represent actively secreting IgG B cells, memory B cells present in the peripheral circulation, or both.

In addition, the process of the present invention has been used to produce B cells making IgG and IgA antibodies. As noted above, γ, α, or μ heavy chains are also expressed on the surface of B lymphocytes. These cells, B cell isotypes, can be isolated using isotype-specific monoclonal antibodies. B cells, isolated from peripheral blood as noted above, are reacted with (FITC)-goat $F(ab')_2$ fragments to human IgG (γ heavy chain), or IgA (α heavy chain), or to IgM (μ heavy chain). The B cells thus labeled with an isotype-specific monoclonal antibody, are then washed and separately applied to the FACS sorter, as described above. B lymphocytes bearing surface γ, α, or μ heavy chains are thus isolated for EBV transformation, culturing and cloning as described in sections "b" and "c" above. See Example 6.

d) Recovering human monoclonal antibodies which specifically bind to a predetermined antigen. The antibodies produced by the process of this invention can be recovered (and concentrated and purified) using standard techniques, e.g. physical-chemical methods. The antibodies are homogenous and are highly specific to the target antigen.

As has been noted above, the human monoclonal antibodies of the present invention are useful in the therapeutic treatment of viral infections, bacterial diseases, tumors and immune modulations, thus eliminating possible foreign reaction response (as is evident in these same therapeutic protocols using murine monoclonal antibodies). These uses are well known to the practitioner in the art (see, for example, the Koprowski patents — 4,172,124; 4,196,265; and 4,349,528). The human monoclonal antibodies of the present invention may be substituted for murine monoclonal antibodies.

The human monoclonal antibodies of the present invention are also useful as diagnostic reagents (similar to murine or other animal-generated reagents) in, for example, ELISA assays, PET assays, and STEM (Scanning Transmission Electron Microscopy) assays. Examples of such use are shown in Burchiel, et al. (Patent No. 4,311,688) and Carlsson, et al. (Pat. No. 4,232,119).

EXAMPLES

Example 1. The binding capacity of biotinylated ligands to B cells was investigated. The B lymphocytes were purified as disclosed in the Specific Disclosure. Human thyroglobulin (Tg) was used as the antigen, and isolated from normal thyroid tissue obtained at autopsy (Roman, et al., Clin. Chem., 30:246 (1984)). Tissue was homogenized in phosphate-buffered saline (PBS, pH 7.2) and centrifuged at 100,000 g, and the supernatant was applied to a Sephadex G-200 column (Pharmacia). The Tg was purified to homogeneity by application of the first eluted peak to a Sepharose 6B column (Pharmacia). Tg was stored in aliquots at -70°C. Tg was labeled with n-hydroxysuccinimidobiotin (Sigma) in 0.1 M carbonate buffer, pH 8.5, at a protein-to-biotin ratio of 4:1, followed by exhaustive dialysis against PBS.

Human B cells were incubated with biotinylated Tg, and after being washed, they were reacted with fluorescein-isothiocyanate (FITC)-avidin. This involved incubating approximately 2×10^7 B cells (5×10^6 per milliliter) for two hours in ice chilled sterile Hank's balanced salt solution with Ca^{2+} and Mg^{2+}, without phenol red, and with 1% bovine serum albumin (BSA-HBSS) containing biotinylated Tg (4.50×10^{-9} M). The cells were washed with cold BSA-HBSS and then allowed to react with FITC-avidin (1.56×10^{-7} M) in cold BSA-HBSS for one hour. A smaller sample of B cells (10^6) was simultaneously incubated with BSA-HBSS devoid of biotinylated Tg and allowed to react with FITC-avidin under similar conditions. After further washing with cold BSA-HBSS, cells from both samples were resuspended at a density of 10^6 per milliliter in the same medium

and at different times applied to a Becton and Dickinson 440 FACS with Argon 466 laser. Approximately 7% of the cells incubated with biotinylated Tg and FITC-avidin displayed a higher degree of fluorescence than their counterparts incubated with FITC-avidin alone. See Figure 1A.

The lymphocytes that bound to the biotinylated Tg were then isolated. Cells were sorted, and the positive fraction and negative fraction were collected and transformed with Epstein-Barr Virus, as described in the Specific Disclosure.

The positively-selected EBV-transformed cells were then resuspended and distributed at 4000 cells per well into a 96-well U-bottom culture plate containing 5×10^4 irradiated (2500 rads) syngeneic human peripheral blood mononuclear cells as a feeder layer. After about 18 days of incubation, culture fluids are harvested and assessed for antibody content. All 48 wells from the positively sorted fraction produced high concentrations of antibody to Tg (95 to 200 ngeq/ml). See Figure 2A and Table 1. In contrast, only 1 of 48 wells from the negatively sorted fraction produced any detectable antibody ($>$ 10 ngeq/ml). Experiments with lymphocytes from three healthy donors yielded similar results. All of the antibodies that reacted with Tg were of the immunoglobulin M (IgG) class.

TABLE 1

Human Monoclonal Antibodies Produced by Sequentially Cloned B Lymphocytes That Had Been Positively Selected for Binding to Biotinylated Tg and Transformed by EBV

Clones	Number of cells per well in sequential cloning steps* I	II	III	Heavy-chain type	Heavy-chain type	Tg-binding activity (ngeq/ml)†
P16 2A1.1	5	10	2	u	k	140
P16 2A1.3	5	5	1	u	k	62
P16 2A1.5	5	10	1	u	k	65
P16 2A1.6	5	2	2	u	k	140
P16 2A1.8	5	5	2	u	k	1200
P16 2A1.9	5	5	1	u	k	920
P16 2A1.13	5	10	2	u	k	105
P16 2A1.14	5	2	2	u	k	75
P16 2A1.15	5	2	2	u	k	65
P16 2A1.16	5	10	2	u	k	160
P16 2A1.18	5	10	2	u	k	500
P16 2A1.19	5	2	2	u	k	80
P16 2A1.20	5	2	2	u	k	65
P16 2A1.27	5	10	2	u	k	130
P33.7+			10		k	200
P32.10+			10		k	200
P33.15+			10		k	200

*Cells per well at which fewer than 20% of the wells containing growing cells. Cells were cloned sequentially three times by liminting dilutions. †Amount of antibody to Tg produced over a 4-week period in the last cloning step. +Characterization of antibodies produced by clones P33.7, P32.10, a P33.15 is included for comparison. These clones were derived from two patients with Hasimoto's disease.

Example 2. To show that the method of the present invention is applicable to other antigens, peripheral blood from healthy donors who had not recently received a booster dose of tetanus toxoid (TT) was obtained from the Commonwealth of Massachusetts (Dept. of Health, Boston, MA) and fractionated to homogeneity by gel filtration on a Sephadex G-150 column (Pharmacia). The B lymphocytes from these donors were incubated with biotinylated TT and subsequently with FITC-avidin, and finally applied to FACS. Approximately 8% of the cells displayed a higher degree of fluorescence than their counterparts incubated with FITC-avidin alone. See Figure 1B. The cells were then sorted and the positive and negative fractions were transformed with EBV and distributed in culture with feeder layers. After 18 days of incubation, the culture fluids were harvested and analyzed for antibody to TT. All 45 wells from the positively sorted fraction produced high concentrations of antibody to TT (100 to 1800 ngeq/ml), whereas only 3 of 48 wells from the negatively sorted fraction produced any detectable antibody (> 10 ngeq/ml). See Figure 2B. All of these antibodies to TT were of the IgM class.

Example 3. To show that antigen-selected, EBV-transformed cell lines actually secrete human monoclonal antibodies, Tg-binding B cells were chosen as exemplary of the present invention. Positively selected-EBV transformed cells of Example 1 were cloned in limiting dilutions at ten, five, two, and one cell per well in the presence of an allogeneic-irradiated feeder layer. Over a period of 4 months, 14 clones were derived from three sequential clonings at different numbers of cells per well (Table 1). Cell lines generated after three cloning steps produced amounts of Tg antibody ranging from 62 to 1200 ngeq/ml. All were IgM antibodies with $\underline{k}$ light chains. The flow cytometry profile of one of the expanded cell clones (clone P16 2A1.3) suggested that virtually all cells bound Tg (Figure 1C), compared with the initial uncloned cell population (Figure 1A).

Example 4. Specific mouse monoclonal antibodies to Leu-1 (CD5) and to human B lymphocytes were used in double fluorescence flow cytometry assays to identify and isolate Leu-1$^+$ B lymphocytes in peripheral blood and spleens from healthy human subjects.

A mononuclear cell fraction enriched in B cells was purified from human peripheral blood by centrifugation through a lymphocyte-separating medium (commercially available from Bionetics, Rockville, MD). Monocytes were removed from mononuclear cells by incubation with carbonyl iron particles, and the iron-loaded monocytes were subsequently removed using a magnet. The mononuclear cells were depleted of T cells by incubation (in ice) with (2-aminoethyl-isothioronium bromide hydrobromide)-treated sheep red blood cells (AET-SRBC). The non-SRBC rosette-forming cells consisted of at least 50% B cells, some residual monocytes and T cells, and a variable number of lymphocytes with the NK phenotype. This non-SRBC rosetting, enriched B lymphocyte fraction is referred to in this Example as B cells.

The B cells were treated with phycoerythrin-conjugated mouse monoclonal antibody (PE-mAB, IgG2a) to B1 (CD20, a B cell marker) and biotin-labeled mAB (biot-mAB, IgG2a) to Leu-1. The cells were washed, incubated with FITC-avidin, washed again, and analyzed by FACS for the presence of B lymphocytes bearing the Leu-1 marker, separating the B lymphocytes into Leu-1$^+$ and Leu-1$^-$ B lymphocytes were then transformed into Ig-secreting cells by infection with EBV. The EBV used in this Example was obtained from a culture fluid of B95-8 marmoset lymphoma cells. This virus preparation had a titer of 5 x 10^5 transforming units per milliliter, one transforming unit being the minimal amount of virus-producing transformation of 10^4 purified human B cells. The positive-selected EBV-transformed cells were then grown as shown in Example 1.

For the production of human monoclonal antibodies, microculture plates were seeded with the EBV-infected cells at various doses and in the presence of irradiated feeder layers (see Table 2). After 4 weeks of culture, fluids were tested for

antibody activity. Enzyme-linked immunosorbent assays were used for the titration of antibodies to the purified Fc fragment of human IgG, ssDNA (single-stranded DNA), or tetanus toxoid (TT). Culture fluids from the EBV-transformed cells were added to the various antigen-coated plates and incubated for 2 hours at room temperature. After the plates were washed with phosphate-buffered saline-Tween-20 (0.05%), peroxidase-conjugated affinity-purified goat $F(ab')_2$ fragment to human IgA, IgD, IgM or IgG was added to different plates allowed to react for 2 hours at room temperature. After further washing, bound enzyme-linked probes were detected by using orthophenylenediamine and H_2O_2 as substrate. The results are shown in Figure 5, where each dot represents the concentration of antibody (expressed as absorbance at 492 nm) in the culture fluid of a single microculture well. Approximately 100 microculture wells were assayed in each column. Table 2 shows that antibodies from each of the major isotypes was produced.

This methodology allows for the identification and segregation of discrete human B cell subsets for the first time. The B cells composing this subset ($Leu\text{-}1^+$) produce antibodies with characteristic binding specificities, similar to those reported for the "natural" antibodies and for certain autoantibodies.

TABLE 2

Ig Class	Leu-1$^+$ Amount of Ig produced	Leu-1$^+$ Fraction producing Ig	Leu-1$^-$ Amount of Ig produced	Leu-1$^-$ Fraction producing Ig	Unfractionated Amount of Ig produced	Unfractionated Fraction producing Ig
IgM	7000±692	1/3	7600±754	1/3	8250±479	1/3
IgG	200± 36	1/70	310± 44	1/55	395± 40	1/60
IgA	71± 11	1/65	77± 32	1/75	105± 7	1/70
IgD	62± 13	1/65	75± 19	1/75	75± 10	1/70

Example 5. B lymphocytes were separated from peripheral blood of healthy individuals as described in Example 1. The B cells were then reacted with FITC-goat $F(ab')_2$ fragment to human IgG (γ heavy chain), IgA (α heavy chain), or to IgM (μ heavy chain). After washing, the B cells were separately applied to a FACS sorter for analysis. As shown in Figure 3, approximately 3.5%, 3.0%, and 60% of lymphocytes reacted with FITC-goat $F(ab')_2$ fragment to γ, α, or μ heavy chains, respectively. B lymphocytes bearing these surface γ, α, or μ heavy chains were then isolated by sorting, infected with EBV, and distributed by limiting dilution into 96 well plates containing 10^5 irradiated syngeneic or allogeneic peripheral blood mononuclear cells as feeders. After 4 weeks in culture, supernatant fluids were analyzed for their Ig content by ELISSA assay. Isotype selection resulted in marked enrichment of the preselected population -- close to 99% of the cells selected for γ, α, or μ heavy chains made IgG, IgA or IgM, respectively.

The supernatants from the isotype selected EBV-transformed B lymphocyte cultures were then tested for antibodies using ELISSA plates coated with purified human thryoglobulin (Tg), recombinant human insulin (Ins), or purified tetanus toxoid (TT). Figures 3 and 4 show that the isotype selection procedure and transformation with EBV resulted in the production of IgG, IgA and IgM antibodies to Tg, Ins, and TT.

Example 6. The EBV-transformed B lymphocytes producing IgG, IgA or IgM antibodies (from Example 5) were fused with cells of a fusion partner, in order to stabilize the cell line. One such fusion partner is described in Pollack, et al., J. Clin. Invest., 1987 (in press) and Larrick, et al., "Human Hybridomas and Monoclonal Antibodies", Plenum Press, NY, pp. 149-165 (1985). Other fusion partners are known to those skilled in the art.

In this Example, EBV-transformed B cells (2×10^7) were fused with the above-mentioned fusion partner (2×10^7) in serum-free RPMI in the presence of 40% polyethyleneglycol

(4,000 M.W.). After fusion, cells were distributed at 2 x 10^5 cells per well in 96 well plates and cultured in selection media (FBS-RPMI containing 10^{-4} M hypoxanthine, 6 x 10^{-5} M azaserine, and 10^{-6} M oubain). Oubain and azaserine were used to select against unfused parental EBV-transformed B lymphocytes and HGPRT deficient human-mouse heteromyeloma cells, respectively. Growth of the human-human-mouse hybrids was generally observed after two weeks. Table 3 shows several cell lines making antibody of the IgG, IgA and IgM class to Tg, Ins, and TT. These clones produced 5-20 ug/ml of IgG, 10-40 ug/ml IgA, and 5-160 ug/ml IgM. Many clones have been expanded in culture for up to six months without alteration in their rate of growth or immunoglobulin secretion. Over 40 antibody-producing clones have now been constructed.

TABLE 3

Human Monoclonal Antibodies Produced by Sequentially Cloned B Lymphocytes Which Had Been Selected For Surface γ, α, or μ Heavy Chains, Transformed With EBV and Fused With F3B6-1A Heteromyeloma

Immunoglobulin Class and Antibody Binding Activity (1)	Clone	Antibody Produced (ug/ml) (2)
Tg		
IgG (k)	P 77.3.3-F1	20.0
IgM (k)	P172.2.1-F7	20.0
IgM (k)	P 57.2.7-F3	160.0
Insulin		
IgG (λ)	P 66.11.2-F2	16.0
IgG (k)	P 32.10-F1	20.0
IgM (λ)	P 86.2.1.1-F18	160.0
IgM (λ)	P 65.1.1-F1	80.0
Tetanus Toxoid		
IgG ( )	P 66.11.2-F5	5.0
IgM (k)	P142.3-F3-F5	140.0

(1) Clones were obtained following four sequential subculturing steps at 0.5 cell/well. After the first, second, third and fourth cloning, antibody of the selected specificity was found in 7, 51, 96 and 100%, respectively of the microculture wells.

(2) Amount of antibody produced within 48 hours to Tg, Ins, and TT in cultures containing 2 to 6 x 10^5 EBV transformed hybrid cells.

While the preferred embodiments of this invention have been disclosed above, those skilled in the art will recognize that modifications may be made without altering the spirit and scope of the invention.

WHAT IS CLAIMED IS:

1. A method of producing an antibody to a particular antigenic determinant comprising:

a) incubating a purified antigen with human B lymphocyte;

b) isolating a B lymphocyte to which said antigen binds;

c) infecting the B lymphocyte isolated in step "b" with Epstein-Barr Virus to form an immortalized cell;

d) culturing said immortalized cell in cell culture media; and

e) recovering said antibody from said culture media.

2. The method of Claim 1 wherein said purified antigen is a human monoclonal antibody.

3. The process of Claim 1 wherein said antigen is thyroglobulin, tetanus toxoid, insulin or B-galactosidase.

4. A process for the production of clones which produce antibodies to a particular antigen comprising:

a) obtaining human B lymphocytes capable of binding to said antigen and infecting said lymphocytes with Epstein-Barr Virus to form an immortalized cell line;

b) cloning cells from said cell line in limiting dilutions; and

c) recovering cloned cells from step "b" which produce antibodies which specifically bind to said antigen.

5. The process of Claim 4 wherein said antigen is biotinylated.

6. The process of Claim 4 wherein said antigen is thyroglobulin, tetanus toxoid, insulin or B-galactosidase.

7. The process of Claim 4 wherein said antigen is a human monoclonal antibody.

8. A method for producing human monoclonal antibodies comprising:

isolating human B lymphocytes of a desired specificity;

immortalizing said B lymphocytes under conditions which will promote extended growth and survival of said B lymphocytes, said immortalizing produces an immortalized cell line;

and isolating human antibodies produced by said immortalized cell line.

9. The method of Claim 8 wherein said B lymphocyte is obtained from the normal human repetoire of B lymphocytes.

10. The method of Claim 8 wherein said B lymphocyte is obtained from humans actively immunized with a foreign antigen or afflicted with a particular disease.

11. The method of Claim 8 wherein said B lymphocyte exhibits a Leu-1^{+} marker.

12. The method of Claim 8 wherein said human B lymphocyte of a desired specificity is produced by incubating an antigen with human B lymphoctyes isolated from human peripheral blood.

13. The method of Claim 8 wherein said human B lymphocyte of a desired specificity are B lymphocyte isotypes, and are isolated by incubating B lymphocytes with isotype-specific antibody probes.

14. The method of Claim 8 wherein said immortalizing step comprises incubating said B lymphocyte with a transforming amount of Epstein-Barr Virus.

15. The method of Claim 8 wherein additionally said immortalized cell line is cultured and cloned.

16. The method of Claim 12 wherein human monoclonal antibodies are produced from said B lymphocyte of a desired specificity, and said monoclonal antibodies specifically bind to said antigen.

17. The method of Claim 13 wherein human monoclonal antibodies are produced which specifically bind to said B lymphocyte isotype.

18. A composition of matter comprising human monoclonal antibodies produced by the process of Claim 4, and media therefore.

19. A composition of matter comprising human monoclonal antibodies produced by the process of Claim 8, and media therefore.

20. A composition of matter comprising human monoclonal antibody produced by the process of Claim 11, and media therefore.

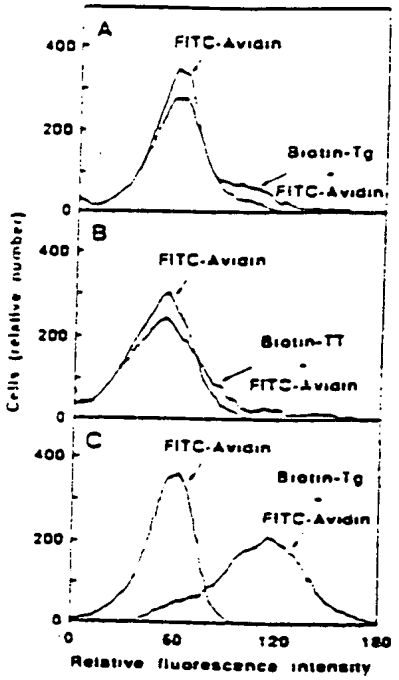
A
FITC-Avidin
Biotin-Tg
FITC-Avidin
B
FITC-Avidin
Biotin-TT
FITC-Avidin
C
FITC-Avidin
Biotin-Tg
FITC-Avidin
Cells (relative number)
400
200
0
0
60
120
180
Relative fluorescence intensity

FIG. 1

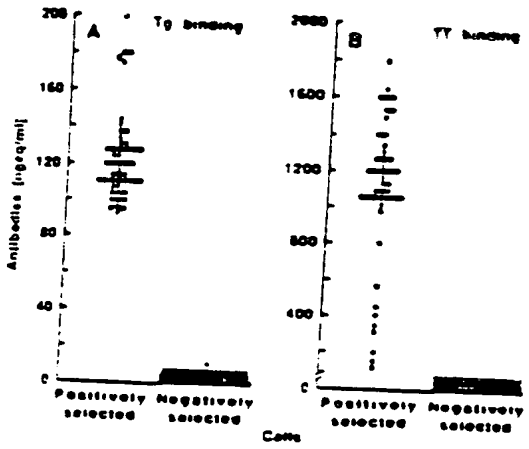
A
Tg binding
B
TT binding
Antibodies (ng eq/ml)
200
160
120
80
40
0
1600
1200
800
400
Positively selected
Negatively selected
Cells

FIG. 2

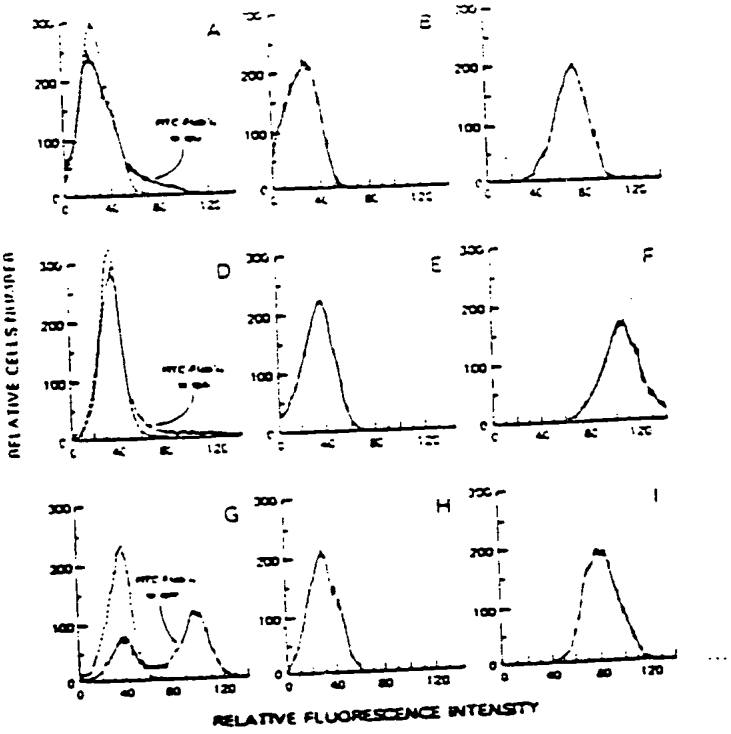
A
B
D
E
F
G
H
I
RELATIVE CELL NUMBER
RELATIVE FLUORESCENCE INTENSITY

FIG. 3

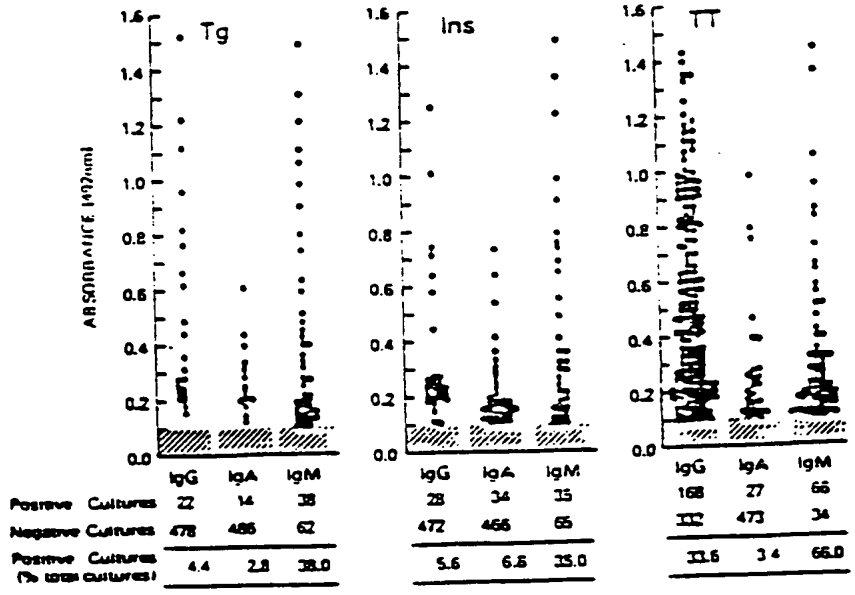
Tg
Ins
TT
ABSORBANCE (492nm)
IgG IgA IgM
Positive Cultures 22 14 38
Negative Cultures 478 486 62
Positive Cultures (% total cultures) 4.4 2.8 38.0
IgG IgA IgM
28 34 15
472 466 65
5.6 6.8 15.0
IgG IgA IgM
168 27 66
332 473 34
33.6 3.4 66.0

FIG. 4

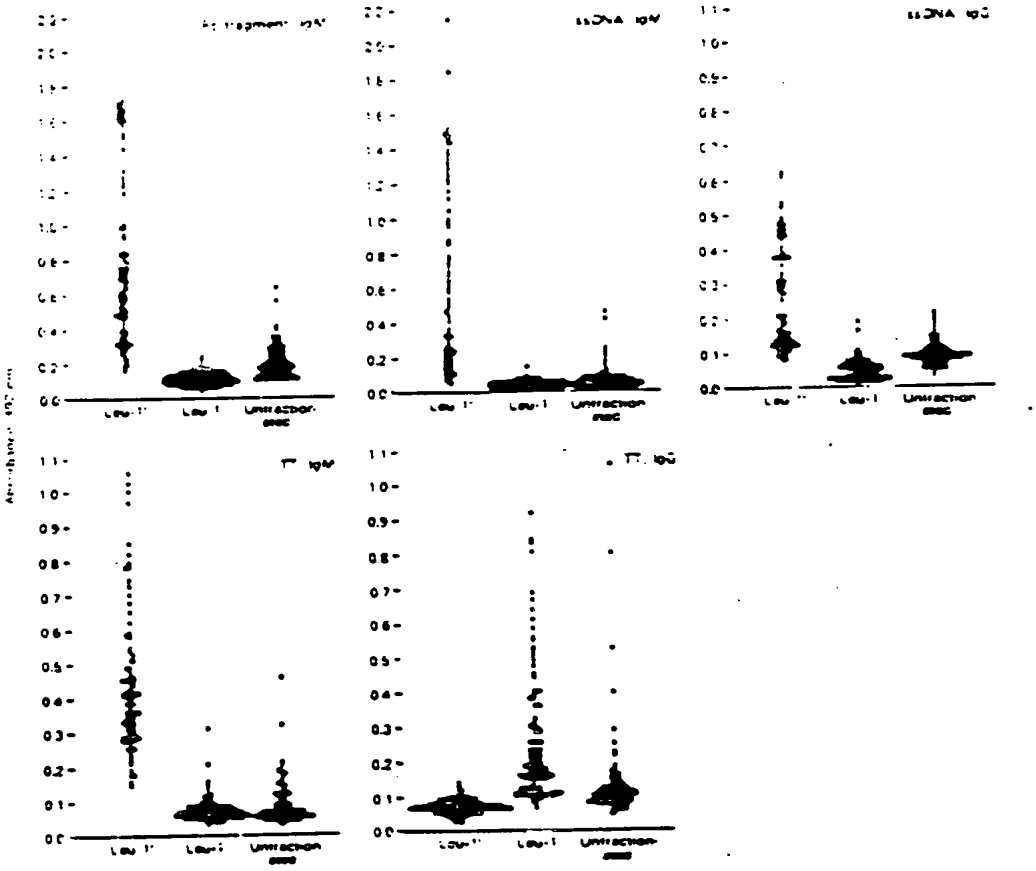

Figure 5

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/02036

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (4): C12P 21/00; C12N 5/00; A61K 39/395
U.S. CL.: 435/68, 240.2; 424/85

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	424/85; 435/68, 172.3, 240.2, 948; 530/387; 935/100

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

DATABASES: AUTOMATED PATENT SYSTEM (FILE USPAT 1975-1988); Chemical Abstracts Online (File CA 1967-1988; File Biosis 1969-1988).

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	Journal of Immunology, Volume 127, No. 4, Issued October 1981, D. KOZBOR ET AL, "Requirements for the Establishment of High-Titered Human Monoclonal Antibodies Against Tetanus Toxoid Using the Epstein-Barr Virus Technique," see abstract pages 1275-1276.	1,3-6, 8,10, 12,14-16, 18,19 2,7,9, 11,13, 17,20
X Y	Journal of Immunology, Volume 135, No. 4, Issued October 1985, P. AMAN ET AL, "Surface Marker Characterization of EBV Target Cells in Normal Blood and Tonsil B Lymphocytes Populations," see pages 2362 and 2363.	1,8,9, 12-14, 16,17, 19 2-7,10 11,15, 18,20

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
19 October 1988	2 1 DEC 1988
International Searching Authority	**Signature of Authorized Officer**
ISA/US	KAY E. CHENEY

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
Y	Journal of Experimental Medicine Volume 153, Issued February 1981, J.A. LEDBETTER ET AL, "Evolutionary Conservation of Surface Molecules That Distinguish T Lymphocyte Helper/Inducer and Cytotoxic/Suppressor Subpopulations in Mouse and Man", see legend to Fig. 2, page 314.	11,20
X Y	Science, Volume 234, Issued 24 October 1986, P. CASALI ET AL, "Human Monoclonals from Antigen-Specific Selection of B Lymphocytes and Transformation by EBV", see abstract, page 476.	1,3-6, 8-10,12, 14-16,18, 19 2,7,11, 13,17,20
X Y	Science, Volume 236, Issued 3 April 1987, P. CASALI ET AL, "Human Lymphocytes Making Rheumatoid Factor and Antibody to ssDNA Belong to Leu-1+ B-Cell Subset", see abstract, page 77.	1,2,4, 7-9,11 12,14-16, 18, 19 3,5,6, 10,13, 17,20
X,P Y	Journal Of Immunology, Volume 140 No. 12, Issued 15 June 1988, NAKAMURA, M. ET AL, "Human Monoclonal Rheumatoid Factor-like Antibodies from CD5 (Leu-1)+ B Cells Are Polyreactive", see page 4180.	1,4,5, 8,9,11, 12,14-16 18-20 2,3,6,7, 10,13,17